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Glomus cubense sp. nov., an arbuscular mycorrhizal fungus from Cuba

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Abstract —*Glomus cubense* (*Glomeraceae, Glomeromycetes*) was isolated from a lagoon vegetation area on a clay soil deposition environment in the vicinity of San José de las Lajas, Cuba. The species description is based on spore morphological parameters from in vivo pot cultures and molecular analyses. The new species is characterized by its small, generally irregular in shape, 20-48 × (24–)54-72 µm hyaline to faintly yellow spores that have a 2-layered spore wall and arise in clusters. Phylogenetic analyses of the rDNA ITS region and H+ATPase place the species into the *Glomeraceae* without close relatives among named *Glomus* species. *Glomus cubense* forms mycorrhizal associations with sorghum and leek plants under greenhouse pot-culture growing conditions and with a diversity of crop plants under field conditions. The name *cubense* refers to Cuba, the country where the species was found.

Keywords — *Glomeromycota*, molecular phylogeny

Introduction

Since 1973, numerous investigations on the diversity of arbuscular mycorrhizal fungi (AMF) have been performed in Cuba, and several species have been described and surveyed from this country (Herrera & Ferrer 1980; Ferrer & Herrera 1981, Herrera-Peraza et al. 2003). With respect to research and development on the selection of AMF strains with potential for use as bio-inoculants for agriculture and horticulture productions, AMF species populations were surveyed and propagated in pure cultures, characterized morphologically, and simultaneously tested for performance at stimulating

plant yields under both controlled and field conditions (Rivera et al. 2003, 2007; Herrera-Peraza et al. 2011). Among the AMF strains surveyed and tested, there was an undescribed species, here named *Glomus cubense*, which formed small, hyaline, 2-layered glomoid spores in clusters around roots. Morphological and molecular comparisons with other light-coloured glomoïd spore species (Błaszkowski et al. 2010) clearly distinguished *G. cubense* from previously described species. Once propagated in pot cultures, it showed high root colonization potential and growth benefits over a variety of crop plants tested. In the present paper, the fungus is proposed as a new arbuscular mycorrhizal species, *G. cubense,* in the *Glomeromycota.*

Materials & methods

AMF strain propagation and evaluation were conducted in the central greenhouse of the National Institute of Agricultural Sciences (INCA) in San José de las Lajas, Cuba. Species morphological and molecular characterizations were done at the Eastern Cereal and Oilseed Research Centre of Agriculture and Agri-Food Canada, Ottawa, Canada.

AMF sampling and propagation

Soil samples were collected at about 50 cm depth from a lagoon area at "San Rafael" Pond in "Las Papas" area (San José de las Lajas, Cuba), with a Haplic Cambisol (chromic) soil (IUSS Working Group WRB, 2006), a natural soil deposition environment occasionally flooded and mainly covered with *Cynodon nlemfuensis* and *Mimosa pigra.* Mixed soil samples were used to establish pot trap-cultures using *Sorghum bicolor* as host plant. After a four months growth period, spores of AMF were isolated by morphotypes and used as inoculant for the establishment of pure cultures in sterilized (121°C for 3 h during three days) clay substrate from a "Gley vértico cálcico" soil (Instituto de Suelos 1999) from "Guayabal" farm in San José de las Lajas, Cuba. Plants were fertilized and watered at regular intervals (Hewitt 1966). Pure strain cultures were subsequently maintained as reference culture on sorghum plants under semi-controlled greenhouse conditions.

AMF isolation and characterization

Spores were extracted from the substrate by wet-sieving (300–38 µm mesh sieves) and centrifugation in sucrose gradient (Dalpé & Hamel 2007). Spores were then manually isolated from the supernatant under the dissecting microscope (Olympus SZ-PT) by micropipetting. Plant roots were washed in water, bleached in 2.5% KOH and stained with fuchsin acid (0.01% in lacto-glycerol) (Kormanik & McGraw 1982). Once destained in lacto-glycerol, root sections were mounted on microscope slides in polyvinyl alcohol-lactic acid-glycerol (PVLG) (Omar et al. 1979), a mixture of PVLG and Melzer's reagent (1:1, v/v), or a mixture of PVLG and Cotton Blue (0.15% in lactic acid) (1:1, v/v). Spore morphology and spore wall architecture were based on examination of about a 100 spores. Color observations referred to the color chart code of the International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi (INVAM; http://invam.caf.wvu.edu/). Microscopic observations were performed with a Nikon Eclipse 800 compound microscope equipped with Nomarski differential

interference contrast optics and photographs were taken with a Nikon CoolPix 4500 digital camera. Nomenclature and AMF classification follow Schüßler & Walker (2010). Reference specimens were deposited at the Cuban National Herbarium, IES-CITMA (HAC) Cuba, and at the National Mycological Herbarium (DAOM) Canada.

Molecular analyses

Spore clusters were collected under a low magnification binocular, rinsed with ddH_2O and air-dried. DNA extraction was performed using the PrepMan Ultra (PMU) reagent (Applied Biosystems). A ratio of 10 spores/µl of PMU reagent were crushed using sterile plastic micropestle. Initial PCR reaction for H^+ ATPase was performed following Sokolski et al. (2010) with primer GmossATPup1058 (5′-CGG TAC TTT GTT CTG ATA AGA C-3′) and primer mix GintraATPlo2663, GmossATPlo2663, GcoroATPlo2663. Three nested PCR were performed using primers pairs:1) GintraATPup1104, GmossATPup1104 with GintraATPlo2663, GmossATPlo2663, GcoroATPlo2663 (Sokolski et al. (2010), 2) *Glomus*ATPup1150 (5′-WTC HGC YGA ACC TGG TGC-3′) with *Glomus*ATPlo2557 (5′-CTK GHT TTW GAW GGC CAK AAT-3′) at 52ºC or 3) with GintraATPlo2663, GmossATPlo2663, GcoroATPlo2663 at 58ºC. The ITS rDNA primers pair used were: GLO2A-forward (5′-CGT AAC AAG GTT TCC GTA GG-3′), GLO2R-reverse (5′-GCG GGT ACT CCT ACC TGA TT-3′)(Sokolski et al. 2010). PCR products were cloned using the TOPO TA Cloning® Kits (Invitrogen, USA). Cloned products were purified using the GenElute™ Plasmid Miniprep Kit (Sigma, USA). Cloning and DNA extraction were performed following the instructions of the manufacturer for rDNA ITS. Between two and eight rDNA ITS molecular clones (av. four) were selected. H+ ATPase and ITS were sequenced using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif.) and run on an ABI 3100-Avant automated sequencer (Applied Biosystems/ Hitachi). Sequence editing was done using SeqMan (DNASTAR, www.dnastar.com/), resulting in 1305 bp and 537, 546 and 549 bp alignments, respectively. The sequences obtained were compared with those in the GenBank databases using the BLAST program. Sequences retrieved from GenBank and those in this study were aligned by Muscle 3.8.31 and a neighbour-joining tree inferred using MEGA 5.0 (Tamura et al. 2011) with 1000 replicates. The best-fit model (General Time Reversible plus Gamma) was selected using the web-based MODELTEST 3.7 (Posada 2006). Sequences were deposited in GenBank under accession numbers JF510464 for H+ ATPase and JF692724, JF692725, JF692726 for ITS rDNA.

Taxonomy

Glomus cubense Y. Rodr. & Dalpé, **sp. nov.** PLATES 1–2

MYCOBANK MB561650

Sporocarpia ignota. Sporae singulares vel aggregatae. Sporae hyalinae vel luteolae, ovoideae, vel irregulares, 20–48 × (24–)54–82 µm, raro globosae, 24–54 µm diam. Sporae tunica stratis duobus; stratum exterius hyalinum, subflexuosum, ad 0.8–1.0 µm crassum, stratum interius hyalinum vel luteolum, 0.8–1.7 µm crassum, rigidum. Hyphae sustinentes hyalinae, rectae, subcylindricae vel subinfundibuliformes, 4–10 µm crassae ad basim sporae. Porus apertus, raro septo curvo clausus cum strato interiore. Mycorrhizas vesicular-arbusculares formantes.

Type: Cuba: "Las Papas" area, San José de las Lajas, from a Fersialítico Pardo rojizo mullido carbonatado soil in "San Rafael" Pond. (**Holotype**, permanent slides mounted in PVLG deposited at HAC Cuban National Herbarium, Instituto de Ecología y Sistemática, CITMA, La Habana, Cuba; **isotype**, DAOM 241198). Genbank JF510464, JF692724, JF 692725, JF692726.

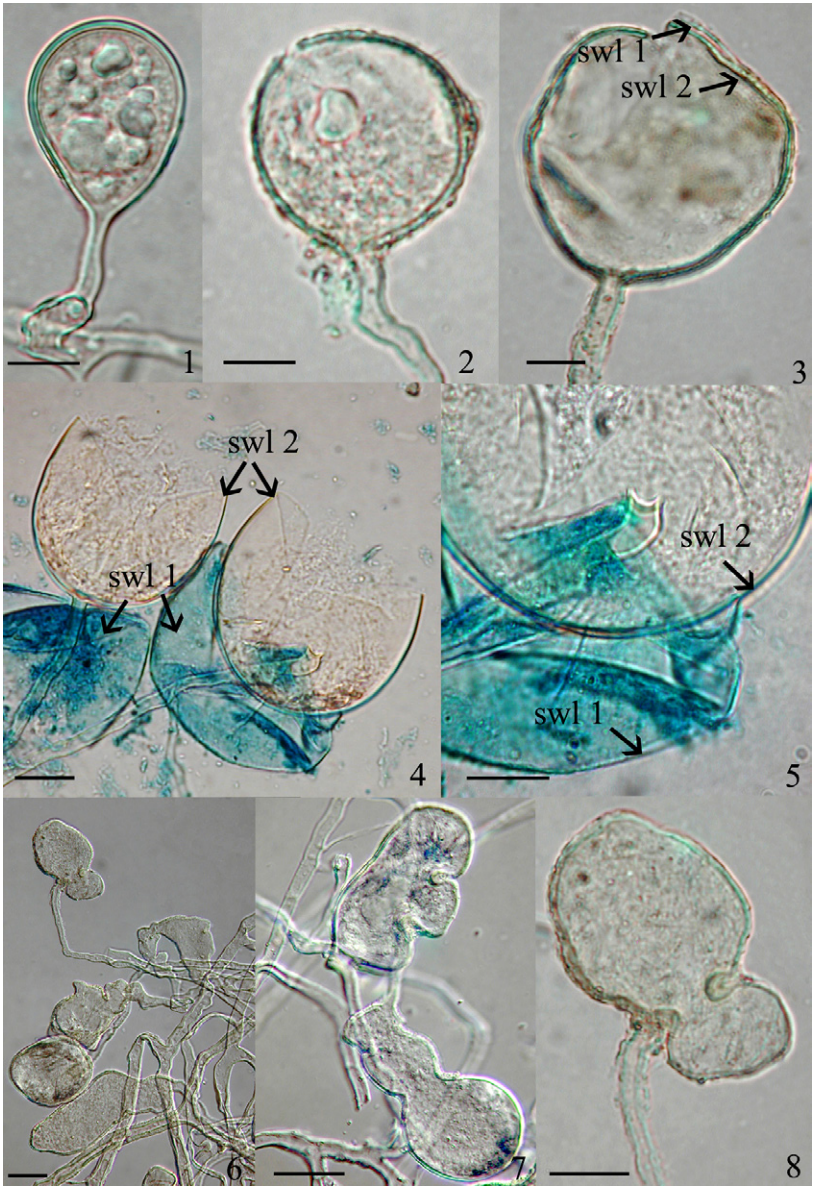
Etymology: Latin, *cubense*, in reference to Cuba, the country where the species was found.

Sporocarp unknown, spores mostly found in loose aggregates of 3 to 12 spores arising blastically at the top of sporogenous hyphae branched from a parent hypha continuous with an extraradical mycorrhizal hypha, rarely single in the soil. Spores (FIGS 1–8) hyaline to very light yellow (0/0/40/0); ovoid, ellipsoid, pyriform to irregular in shape 20–48 × (24–)54–82 µm, rarely globose, 24–54 µm in diameter with one subtending hypha. Spore wall composed of two layers. Spore wall layer 1 (Swl 1) hyaline, permanent, flexible to semi-flexible, 0.8–1.0 µm thick, with a smooth surface, easily separating from layer 2 in crushed spores. Swl 2 hyaline to very pale yellow, rigid, 0.8–1.7 µm thick. Spore wall layers do not react to Melzer's reagent, spore wall layer 1 stained light blue in Cotton Blue. Subtending hypha concolorous to spores; cylindrical to slightly flared, straight or recurved; 4–10 µm wide at spore base. Wall of subtending hyphae hyaline to very light yellow; 1.6–2.0 µm thick at the spore base; composed of two layers continuous with spore wall layers 1 and 2. Pore usually open, occasionally closed by a curved septum located 6–8 µm from the spore base or sometimes occluded by thickening of spore wall layer 2.

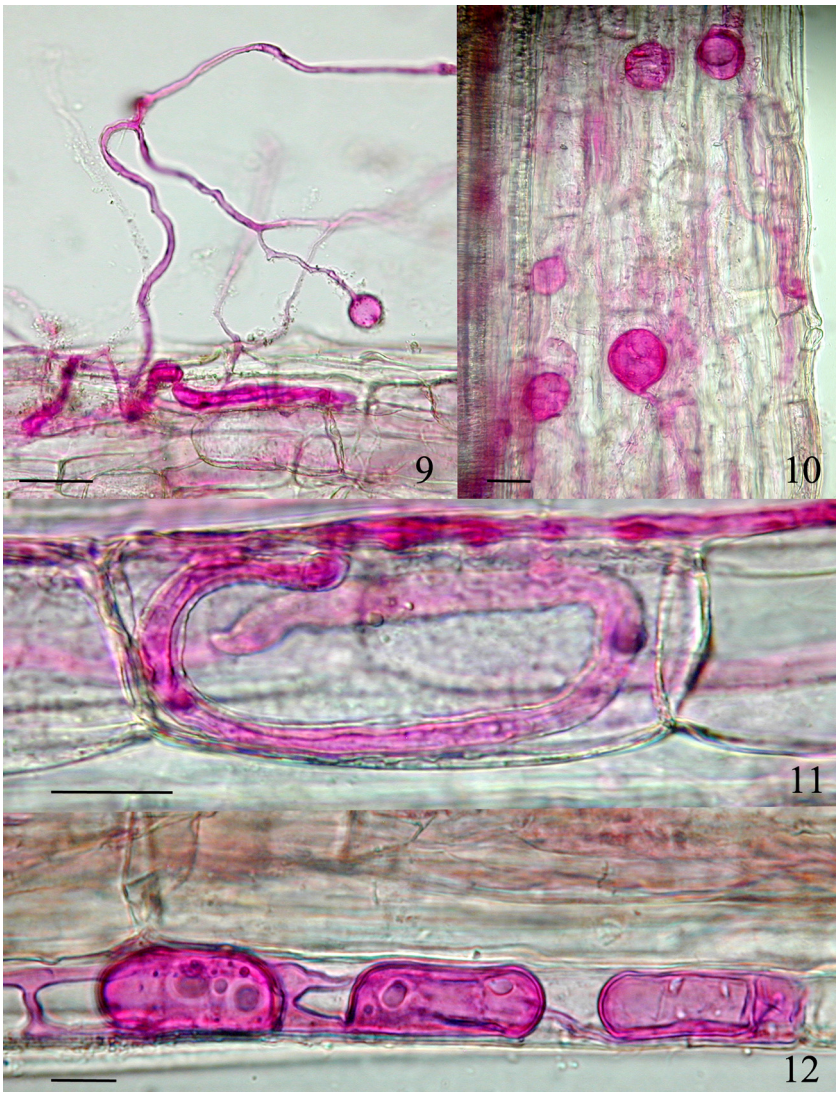
Intraradical structures — *G. cubense* forms vesicular-arbuscular mycorrhizae with intracellular ellipsoid hyphal coils (FIG. 11), globose, 23–38 µm, to ovoid to irregular vesicles, 18–25 × 20–60 µm (FIGS 10, 12) and a few arbuscules.

Mycorrhizal association — In the field, found associated with typical lagoon vegetation plants such as *Cynodon nlemfuensis* and *Mimosa pigra.* In field trials, developed symbiosis with a diversity of crop plants: avocado trees, banana, cassava, corn, cucumber, forages (*Brachiaria decumbens* and *Panicum maximum*), guava, malanga, mango, pepper, plantain, potato, rice, soybean, bean, sweet potato, tobacco, tomato, wheat, yam (Rivera et al. 2007). In the greenhouse, pot cultured for several years with sorghum (*Sorghum bicolor* and leek (*Allium porrum*) plants.

Phylogenetic position — The ITS rDNA and H+ ATPase analyses (Figs 13–14) placed *Glomus cubense* in the genus *Glomus* sensu Schüβler & Walker (2010) in the family *Glomeraceae* with no close relationship with known AMF species of this family. Moreover, no environmental sequences were found to be closely related to *G. cubense*.



Figs. 1–8. Spores of *Glomus cubense* (DIC) from pot-cultures. 1. Juvenile spore with hyphal attachment. 2. Mature spore with hyphal attachment. 3. Mature spore slightly crushed showing spore wall layers 1 and 2 (swl1, swl2). 4–5. Mature spores with spore wall layer 1 stained with Cotton Blue compared to spore wall layer 2. 6–8. Irregular shaped spores with subtending hyphae. Bars: 1–3 =10 µm; 4–8 = 20 µm.



Figs. 9–12. *Glomus cubense* fungal structures inside colonized roots (DIC). 9. Entry point along a root. 10. Vesicles. 11. Hyphal coil inside cortical root cell. 12. Vesicles inside cortical root cell. Bars: 9–12 = 20 µm.

Specimens examined: **CUBA.** Spores and roots isolated from S*orghum bicolor* pot cultures, grown in greenhouse on sterilized clay substrate (Gleyic Calcic Vertisol soil (IUSS Working Group WRB, 2006) with the following chemical characteristics: Na^+ 0.81, K^+ 0.42, Ca^{2+} 48.1, Mg^{2+} 6.6 (cmol/kg^{-1}); P 30.8 (ppm); organic matter 0.84 (%); pH

8.3. **CANADA.** Spores and roots isolated from *Allium porrum* pot cultures, grown on cultivated Brown Chernozem soil (Saskatchewan, Canada) with a loamy sand texture and the following chemical characteristics after sterilization: NH_4-N 19.7 and NO_3-N 14.1 (mg. kg^{-1}), P 21.3 and K^+ 324.5 (mg. kg^{-1}), pH 6.5 and EC 0.48 mS.

Other specimens examined: Type specimens of *G. bistratum* Błaszk. et al., *G. perpusillum* Błaszk. & Kovács, provided by J. Błaszkowski, *G. canadense* (Thaxt.) Trappe & Gerd. (FH 5045), *G. fragile* (Berk. & Broome) Trappe & Gerd. (K Berkeley 1182) and of *G. viscosum* T.H. Nicolson (PI Holotype)

Distribution and habitat. *Glomus cubense* is only known from a single location: "Las Papas" area, San José de las Lajas, Cuba (22°57′41″N 82°09′04″W). Spores were originally isolated from Haplic Cambisol (chromic) soil collected at ca. 50 cm depth. The soil corresponds to a "Fersialítico Pardo rojizo mullido carbonatado" soil (Instituto de Suelos, 1999). The species was propagated in pot-culture using the same type of soil originating from the same area in Cuba.

Notes

The main distinctive morphological properties of *G. cubense* are its small hyaline to very light yellow, usually irregularly-shaped spores (Figs 1–2, 6–8) and its 2-layered spore wall (Figs 3–5) composed of a permanent, flexible to semi-flexible outer layer staining distinctly in Cotton Blue (Figs 4–5) and a rigid inner layer, non-reactive to Melzer's reagent. Under the dissecting microscope, *G. cubense* may resemble most glomoid small-sized and pale-colored spores as treated in the Błaszkowski et al. (2010) identification key. However, when observed under the compound microscope, only six named species produce glomoid pale coloured, inamyloid and 2-layered spores that are smaller than 80 µm. These are *G. bistratum* (Błaszkowski et al. 2009b), *G. cerebriforme* McGee (McGee 1986), *G. indicum* Błaszk. et al. (Błaszkowski et al. 2010), *G. minutum* Błaszk. et al. (Błaszkowski et al. 2000), *G. pallidum* I.R. Hall (Hall 1977; Oehl et al. 2003), and *G. perpusillum* Błaszk. & Kovács (Błaszkowski et al. 2009a).

The *G. cerebriforme* and *G. perpusillum* spore wall layer 1 is laminate (vs. flexible in *G. cubense*) and much thicker than spore wall layer 2 (vs. opposite in *G. cubense*). Additionally, spore wall layer 2 of *G. perpusillum* stains in Melzer's reagent (vs. no staining in *G. cubense*). In the spore wall of the other species listed above, layer 1 is a permanent structure, semi-flexible to rigid, thinner than layer 2 as in *G. cubense* but layer 2 is laminate. The *G. minutum* spores are only hyaline and of globose to subglobose shape (vs. hyaline to very pale yellow, and mostly of irregular shape in *G. cubense*). While spore wall layer 1 easily separates from spore wall layer 2 in freshly crushed *G. cubense* spores, that of *G. minutum* generally remains adherent to the laminate spore wall 2. In *G. minutum*, spore wall layer 1 sometimes swells and separates from spore wall layer 2, but only when spores are mounted in lactic acid-based mountants for at least some hours.

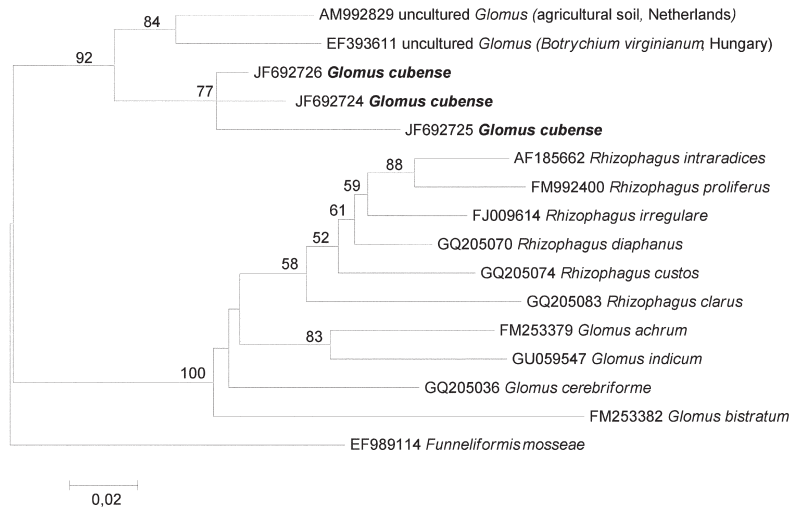


Fig.13. BIONJ tree based on an alignment of the ITS sequences obtained from *Glomus cubense* and related representative sequences from GenBank. Branch support values (only values exceeding 50% are given) on branches refer to BIONJ bootstrap (using GTR + G distances) and maximum likelihood. Scale indicates nucleotide substitution per site.

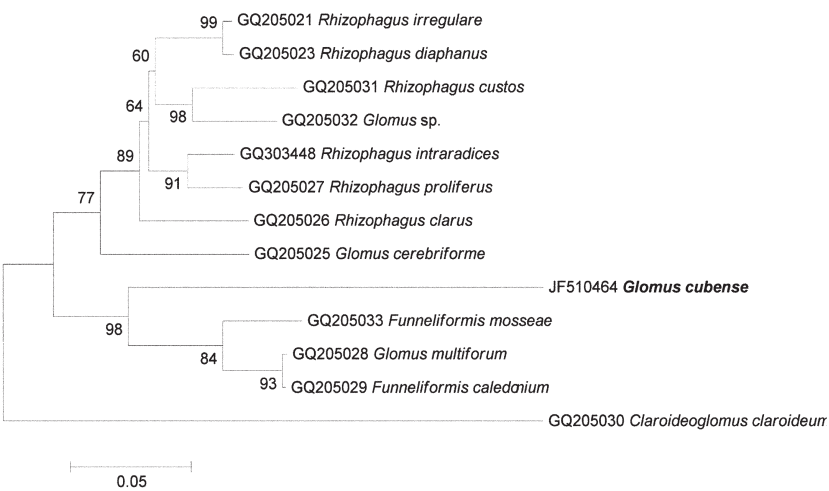


Fig.14. BIONJ (Neighbour-joining) tree based on an alignment of H+ ATPase sequences of *Glomus cubense* and of related representative sequences from GenBank. Branch support values (only values exceeding 60% are given) on branches refer to BIONJ bootstrap (using GTR + G distances) and maximum likelihood. Scale indicates nucleotide substitution per site.

Glomus microaggregatum Koske et al. also produces small, 2-layered spores frequently irregular in shape, but the spores are much darker-colored (yellow to brownish yellow), their spore wall is thicker (up to 4 µm vs. up to 2.7 in *G. cubense*) and comprises a rigid outer layer and a flexible inner layer (vs. flexible to semi-flexible outer layer and rigid inner layer in *G. cubense*).

The spore wall of *G. fragile* and *G. canadense* also consists of a hyaline outer layer and a pale yellow inner layer. However, observation of their respective type specimens (K Berkeley 1182, FH 5045) revealed that the inner spore wall layer in both species is thicker than that of *G. cubense* and laminate (Gerdemann & Trappe (1974).

Young or small-sized clustered spores of *Glomus viscosum* may resemble those of *G. cubense,* but they usually are globose, have a 3-layered spore wall, of which layer 3 is distinctively laminate.

Compared with *G. cubense,* glomoid spores of *Paraglomus* spp are hyaline to pale cream, they form only singly (vs. in cluster in *G. cubense*) and their spore wall layers clearly differ phenotypically from those of *G. cubense* (Błaszkowski et al. 2010).

Ambispora spp. differentiate hyaline but much larger spores (Walker et al. 2007).

The phylogenetic analyses of sequences of the H+ATPase gene clearly support morphological analyses and separate *G. cubense* from the few sequences actually available and confirmed the efficiency of earlier published primers for sequencing *Glomus* species (Sokolski et al. 2010). The analyses of ITS region confirm that the species *G. cuben*se is a member of the genus *Glomus* sensu Schüβler & Walker (2010) in the family *Glomeraceae* without having any close described relatives. When comparing ITS sequence data, the best Blast alignment with Genbank database sequences indicated a maximum of 83 and 85% similarity with an as yet uncultured *Glomus* sp. thus supporting the status of *G. cubense* as a distinct species.

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